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Sofia Martins Farinha

Development of resistance to clinical antifungals by *Aspergillus fumigatus* – a side effect of agricultural antifungals?

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I want to dedicate this work to my parents, who invested in my education and have always believed in me.

Development of cross-resistance by *Aspergillus fumigatus* to clinical azoles following exposure to prochloraz, an agricultural azole.

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Abstract

Background: The purpose of this study is to unveil whether azole antifungals used in agriculture, similar to the clinical azoles used in humans, can evoke resistance among relevant human pathogens like *Aspergillus fumigatus,* an ubiquitous agent in nature. Additionally, cross-resistance with clinical azoles was investigated. Antifungal susceptibility testing of environmental and clinical isolates of *A. fumigatus* was performed according to the CLSI M38-A2 protocol. *In vitro* induction assays were conducted involving daily incubation of susceptible *A. fumigatus* isolates, at 35ºC and 180 rpm, in fresh GYEP broth medium supplemented with Prochloraz (PCZ), a potent agricultural antifungal, for a period of 30 days. Minimal inhibitory concentrations (MIC) of PCZ and clinical azoles were monitored every ten days. In order to assess the stability of the developed MIC, the strains were afterwards sub-cultured for an additional 30 days in the absence of antifungal. Along the *in vitro* induction process, microscopic and macroscopic cultural observations were registered.

Results: MIC of PCZ increased over 32 times in just ten days after initial PCZ exposure; cross-resistance to all tested clinical azoles was observed. The new MIC value of agricultural and of clinical azoles maintained stable in the absence of the selective PCZ pressure. PCZ exposure was also associated to morphological colony changes: macroscopically the colonies became mostly white, losing the typical pigmentation; microscopic examination revealed the absence of conidiation.

Conclusions: PCZ exposure induced *Aspergillus fumigatus* morphological changes and an evident increase of MIC value to PCZ as well as the development of cross-resistance with posaconazole, itraconazole and voriconazole.

Keywords: Aspergillus fumigatus; Cross-resistance; Clinical and agricultural azoles

Background

The ubiquitous saprophytic mould *Aspergillus fumigatus* is known to cause a spectrum of diseases in humans, including allergic syndromes, noninvasive infections, as well as invasive aspergillosis, a condition associated with significant morbidity and mortality [1]. *A. fumigatus* is one of the potentially human pathogenic fungi that have a natural habitat in the environment, including soil and plants [2]. Some members of the azole drug class, which includes voriconazole and posaconazole have been shown to be effective in the treatment of invasive aspergillosis [3]. While azole resistance among clinical *A. fumigatus* isolates has for long been considered to be an uncommon finding, recently multiazole resistance has been reported to be emerging and is increasingly recognized as a cause of clinical treatment failure [4,5]. In agriculture, thousands of tons of azoles are sold annually for the purpose of plant protection, either to control fungal growth that can cause extensive loss of crops or to prevent, or to ease the problem of postharvest spoilage of plants and fruits [6]. All azoles compounds mode of action - irrespectively of their chemical structure and variable biological properties - is based on the interference with the activity of fungal lanosterol 14 alpha-demethylase; such enzyme is responsible for the transformation of lanosterol in ergosterol, an essential constituent of the fungal cytoplasmatic membrane which is encoded by *Cyp51A* gene in *A. fumigatus*. The inhibition of ergosterol formation results in cell wall disorganization and, finally, the impairment of fungal growth. The mode of action of azoles is, therefore, fungistatic rather than fungicidal. It is well known that a strong and persistent antimicrobial pressure can lead to the selection of resistant clones within a microbial population, particularly if the drug effect is static rather than microbicidal [7]. While fungal diseases are problematic for both human health and agriculture (vegetable and animal production), the azole class of drugs is the core therapy both for human and

agricultural fungal diseases. A major concern with this fact is that it may represent an early step in the emergence of *A. fumigatus* resistant isolates, driven by the extensive use of azole fungicides in agriculture, with the same mode of action as azoles used in humans [7,8,9,10]. Evidence about an environmental route of resistance is gaining expression [9,10]; findings suggest that it may be responsible for cross-resistance expressed by clinical and environmental isolates of *A. fumigatus* across several countries [11]. The aim of our study was to investigate whether PCZ, a fungicide extensively use in agriculture, could be associated with the emergence of cross-resistance with clinical azoles amoung *A. fumigatus*.

Methods

Organisms

Two clinical isolates of *A. fumigatus,* LMF05 and LMF11, and one environment *A. fumigatus* isolate (LMN60, recovered nearby the hospital), were used in this study. The isolates were identified as belonging to *A. fumigatus* species by macroscopic and microscopic morphology, the ability to grow at 48ºC and by using MALDI-TOF MS to accurately discriminate *A. fumigatus* from a new sibling species *A. lentulus*, which cannot be distinguished by morphological characteristics or growth peculiarities [12]. Long-term preservation of conidial suspensions of the isolates was made in a GYEP medium (2% glucose, 0.3% yeast extract, 1% peptone) broth supplemented with 10% glycerol and stored at -80◦C. Working cultures were subsequently maintained during 2 weeks on Sabouraud dextrose agar slants and plates at 4°C.

Antifungal agents and susceptibility profile

Prochloraz (PCZ) was used as a representative of agriculture azoles; Fluconazole (FLC), Voriconazole (VCZ) Posaconazole (POS) and Itraconazole (ITZ) as selected clinical azoles. Prochloraz was ressuspended in 80% acetone solution at a final concentration of 5 mg/L. Clinical azoles were dissolved in dimethysulphoxide (DMSO) to obtain stock solutions of 10 mg/L. All drugs were stored at -20ºC. Broth microdilution susceptibility assay was performed according to the Clinical and Laboratory Standards Institute M38-A2 protocol in order to evaluate the initial minimal inhibitory concentrations (MIC) of PCZ and of all the clinical azoles [13]. Drug concentration ranged from 0.125 to 64 mg/L of FLC and PCZ; and 0.0313 to 16 mg/L of POS, VCZ and ITZ. FLC was used as control, since *A. fumigatus* shows a non-susceptible phenotype and MIC is most often above 64 mg/L for this species. MIC of azoles was defined as the lowest concentration of the drug that produced no visible growth following 48 hours of incubation. MIC determination was repeated at least twice.

In vitro induction experiments

Induction experiments were performed with the agricultural azole PCZ. *A. fumigatus* isolates were grown on Saboraud dextrose agar at 37ºC for 72h; conidia were harvested by flooding the surface of the slants with phosphate-buffered saline (PBS) containing 0.025% (vol/vol) tween 80 while gently rocking. The conidial suspensions were then adjusted using specific spectrometric readings at 550 nm to a final concentration of $5x10^4$ conidia per militer [14]; one militer of each distinct isolate suspension was transferred to 9 ml of GYEP broth supplemented with sub-inhibitory concentrations of PCZ and incubated overnight at 37ºC with agitation (180rpm). Daily, after vigorous vortexing for 60 seconds, one militer from each culture was transferred to fresh GYEP medium supplemented with PCZ at the concentration of 0.25 mg/L; in parallel, 1 ml of

the culture was added with 10% glycerol and frozen at -80ºC. This procedure was repeated along thirty consecutive days.

Susceptibility testing/ Stability of *in vitro* developed resistance phenotype

MICs of PCZ were determined every five days along the thirty days of induction assay. No official breakpoints are yet defined for PCZ; therefore, whenever a marked MIC increase was observed (four fold the initial PCZ MIC), the MIC values of clinical antifungals were determined.

In order to assess the stability of the developed MIC increment to PCZ and of the developed cross-resistance to clinical azoles, the induced strains were afterwards sub-cultured for an additional thirty days in the absence of the drug and MIC values re-determined, as previously described.

Culture macro and micro morphology

Every two days along the induction process, from the successive cultures, a loopful was inoculated in Saboraud Agar slants to check for viability and purity of culture; the macro and microscopical growth characteristics were registered; colony morphology and pigmentation were recorded photographically using a Reflex Nikon D3200 Camera and images were processed by Adobe Photo Deluxe Image Processing Program (San Jose, CA, USA).

Results

In vitro induction assay

The 3 isolates developed a progressive increment of PCZ MIC value comparatively to the initially determined value. In addition, a concomitant increase of the MIC of VCZ,

POS and ITZ, was observed (Table 1). From day 0 of evaluation until day 30 during the induction assay, MIC of PCZ increased 256 times. Concerning the clinical azoles, cross-resistance was developed for all days; all isolates changed from a susceptible to a resistant phenotype, according to Meletiadis and colleagues [15].

Macroscopical and microscopical growth changes during the induction assay

Macroscopic and microscopic morphological alterations were observed following exposure of *A. fumigatus* to PCZ. During the induction assay, initially it was notice a macroscopic modification of the pigmentation of *A.fumigatus* colonies, changing from the initial green colour to white (figure1). With the progressive increase of MIC values of PCZ, colonies of *A. fumigatus* expressed dramatic changes, resulting in totally white, scanty and sparse colonies. Microscopic examination in detail of such white colonies showed almost complete absence of conidiation, the white mycelia corresponding solely to hyphae.

Stability of the *in vitro* developed resistant phenotype

The *in vitro* developed high MIC values of PCZ maintained stable following removal of the selective pressure of the drug. For VRC, the MIC value decreased only after 30 days of incubation without the selective pressure, changing the susceptibility phenotype from resistant to intermediate. For POS, the developed MIC value also decreased but not enough to change the phenotype of resistance. Regarding ITZ, it was observed the complete reversibility of the resistant phenotype in the absence of PCZ, ie, the MIC reverted to the initial value and that value is categorize as susceptible. Following

removal of the selective pressure of PCZ, conidiation reappeared together with the green typical color of mature colonies, in all isolates.

Discussion

While very few studies have characterized azole resistance in *A. fumigatus,* some addressed the possible cross-resistance between environmental and medical azoles [8,9,10,11]. Our study demonstrated the time frame between the introduction of a widely used agricultural fungicide and the emergence of cross-resistance to medical triazoles. The exposure of other clinical relevant moulds to agricultural azoles might therefore be associated with the emergence of cross-resistance to clinical antifungals. Besides the emergence of cross-resistance, we found that PCZ exposure caused marked morphological colony changes, both at macroscopical level (the colonies turned white losing the characteristical pigmentation) and microscopic: detailed examination revealed the absence of conidia. Changes in the pigmentation of *A. fumigatus* colonies and the absence of conidia as consequence of azoles' effect have already been reported [16]. Notably, such morphological changes may be responsible for laboratorial diagnostic misidentification of the fungal genus/species, following its initial isolation, with the inherent consequences.

The possibility of development of *A. fumigatus* clones resistant to medical triazoles stresses the need to find safer alternative compounds to agriculture azoles. Since PCZ was responsible for the emergence of stable resistance to itself and to very important medical triazoles in *A. fumigatus*, a resistance mechanism might have been developed. Previous reports describe cyp51A mutation, efflux pump overexpression and/or target upregulation the main mechanisms responsible for such resistance [17,18,19]. Certainly, our next study is to unveil the underlying molecular mechanism. Meanwhile, our study

198 strongly suggests that the abuse of environmental azoles might cause serious veterinary
199 and human health problems in particular to immunocompromised patients, a high risk
200 population for invasive aspergilosis. Azole-resistance and cross-resistance has the
201 potential to further compromise the efficacy of clinical azoles in the future [4,19,20,21].
202 Very recently, several compounds were screened in order to find new antifungal
203 alternatives preventing the possible loss of efficacy of clinical azoles [22].
204

205 **Conclusions**

206 In order to assess the real dimension of *Aspergillus* resistance, a susceptibility test
207 should be performed in all isolates from patients with *Aspergillus* infection. Moreover,
208 initial combination therapy may be considered, in geographical areas with high
209 prevalence of environmental azole resistant isolates, for patients with severe infection.
210 Ultimately, surveillance studies in both clinical settings and in the environment should
211 be conducted in order to provide updated local data regarding susceptibility profiles.
212

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221

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Figure 1 – Photographs of Sabouraud dextrose agar plates showing macroscopic morphological changes of colonies of *A. fumigatus* following exposure to subinhibitory concentration of PCZ.

A. Initial morphological aspect (control).

B. After fifteen days.

C. After thirty days.

Table 1 Susceptibility pattern of tested *A.fumigatus* isolates to Prochloraz and clinical azoles.

***A. fumigatus* isolate**	**Time of exposure (days)**	MIC (mg/L)				
		PCZ	**VRC**	**POS**	**ITZ**	**FLC**
LMF05	**0**	0.125	0.125	0.25	2	>64
	10	0.25	0.25	0.5	2	>64
	20	8	2	1	4	>64
	30	32	8	2	8	>64
	Ø30	32	2	2	2	>64
LMF11	**0**	0.125	0.25	0.125	0.5	>64
	10	0.125	2	0.25	1	>64
	20	8	8	1	2	>64
	30	32	>16	4	4	>64
	Ø30	32	2	1	0.5	>64
LMN60	**0**	0.25	0.25	0.125	0.25	>64
	10	4	8	0.25	1	>64
	20	8	8	0.5	2	>64
	30	64	>16	4	4	>64
	Ø30	64	2	1	0.25	>64

PCZ= Prochloraz; VCZ= Voriconazole; POS= Posaconazole; ITZ= Itraconazole; FLU= Fluconazole; Ø= MIC after 30 days of culture in the absence of PCZ.

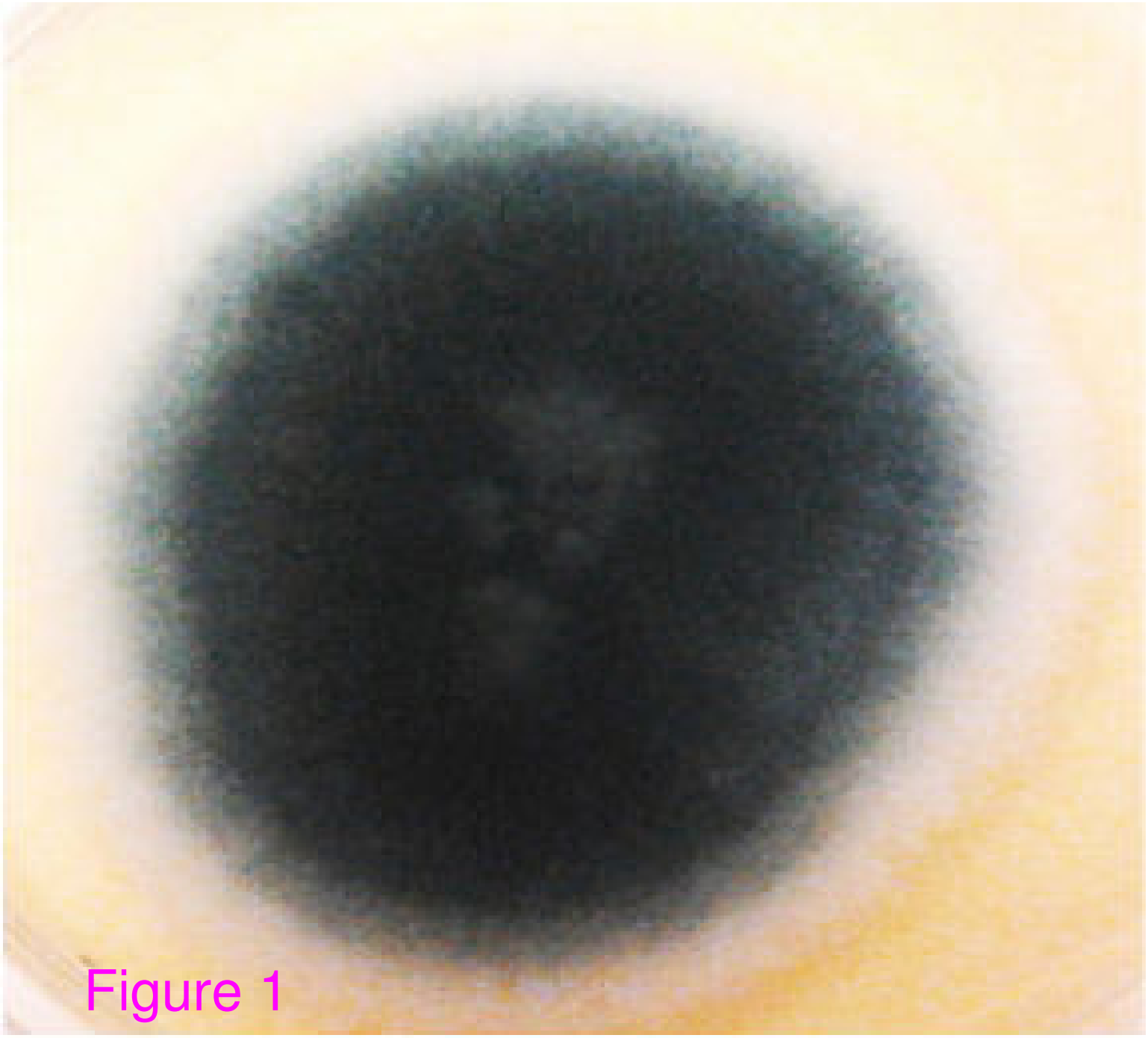

Figure 1

Figure 2

Figure 3

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- Abstract
- Keywords
- Background
- Results and discussion
- Conclusions
- Methods (can also be placed after Background)
- Availability of supporting data
- List of abbreviations used (if any)
- Competing interests
- Authors' contributions
- Authors' information
- Acknowledgements
- Endnotes
- References
- Illustrations and figures (if any)
- Tables and captions
- Preparing additional files

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Book chapter, or article within a book Schnepf E: **From prey via endosymbiont to plastids: comparative studies in dinoflagellates.** In *Origins of Plastids. Volume 2.* 2nd edition. Edited by Lewin RA. New York: Chapman and Hall; 1993:53-76.

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Whole conference proceedings Smith Y (Ed): *Proceedings of the First National Conference on Porous Sieves: 27-30 June 1996; Baltimore.* Stoneham: Butterworth-Heinemann; 1996.

Complete book Margulis L: *Origin of Eukaryotic Cells.* New Haven: Yale University Press; 1970.

Monograph or book in a series Hunninghake GW, Gadek JE: **The alveolar macrophage.** In *Cultured Human Cells and Tissues.* Edited by Harris TJR. New York: Academic Press; 1995:54-56. [Stoner G (Series Editor): *Methods and Perspectives in Cell Biology,* vol 1.]

Book with institutional author Advisory Committee on Genetic Modification: *Annual Report.* London; 1999.

PhD thesis Kohavi R: **Wrappers for performance enhancement and oblivious decision graphs.** *PhD thesis.* Stanford University, Computer Science Department; 1995.

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